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SARA, a FYVE Domain Protein that Recruits Smad2 to the TGFβ Receptor

Tomoo Tsukazaki,* Theodore A. Chiang,*†
Anne F. Davison,*† Liliana Attisano,‡
and Jeffrey L. Wrana*†§
*Program in Developmental Biology
The Hospital for Sick Children
Toronto, Ontario
Canada M5G 1X8
†Department of Medical Genetics and Microbiology
‡Department of Anatomy and Cell Biology
University of Toronto
Toronto, Ontario
Canada M5S 1A8

Summary

Smads transmit signals from transmembrane ser/thr kinase receptors to the nucleus. We now identify SARA (for *S*mad *a*nchor for *r*eceptor *a*ctivation), a FYVE domain protein that interacts directly with Smad2 and Smad3. SARA functions to recruit Smad2 to the TGFβ receptor by controlling the subcellular localization of Smad2 and by interacting with the TGFβ receptor complex. Phosphorylation of Smad2 induces dissociation from SARA with concomitant formation of Smad2/Smad4 complexes and nuclear translocation. Furthermore, mutations in SARA that cause mislocalization of Smad2 inhibit TGFβ-dependent transcriptional responses, indicating that the regulation of Smad localization is important for TGFβ signaling. These results thus define SARA as a component of the TGFβ pathway that brings the Smad substrate to the receptor.

Introduction

Members of the transforming growth factor β (TGFβ) superfamily signal through a family of cell-surface transmembrane serine/threonine kinases, known as type I and type II receptors (Heldin et al., 1997; Attisano and Wrana, 1998; Kretzschmar and Massagué, 1998). Ligand induces formation of heteromeric complexes of these receptors, and signaling is initiated when receptor I is phosphorylated and activated by the constitutively active kinase of receptor II (Wrana et al., 1994). The activated type I receptor kinase then propagates the signal to a family of intracellular signaling mediators known as Smads.

Three classes of Smads with distinct functions have been defined: the receptor-regulated Smads, which include Smad1, 2, 3, 5, and 8; the common mediator Smad, Smad4; and the antagonistic Smads, which include Smad6 and 7 (Heldin et al., 1997; Attisano and Wrana, 1998; Kretzschmar and Massagué, 1998). Receptor-regulated Smads (R-Smads) act as direct substrates of specific type I receptors, and the proteins are phosphorylated on the last two serines at the carboxyl terminus within a highly conserved SSXS motif (Macías-Silva et al., 1996; Abdollah et al., 1997; Kretzschmar et al., 1997; Liu et al., 1997b; Souchelnytskyi et al., 1997). Regulation of R-Smads by the receptor kinase provides an important level of specificity in this system. Thus, Smad2 and Smad3 are substrates of TGFβ or activin receptors and mediate signaling by these ligands (Macías-Silva et al., 1996; Liu et al., 1997b; Nakao et al., 1997), whereas Smad1, 5, and 8 are targets of BMP receptors and propagate BMP signals (Hoodless et al., 1996; Chen et al., 1997b; Kretzschmar et al., 1997; Nishimura et al., 1998). Once phosphorylated, R-Smads associate with the common Smad, Smad4 (Lagna et al., 1996; Zhang et al., 1997), and mediate nuclear translocation of the heteromeric complex. In the nucleus, Smad complexes then activate specific genes through cooperative interactions with DNA and other DNA-binding proteins such as FAST1, FAST2, and Fos/Jun (Chen et al., 1996, 1997a; Liu et al., 1997a; Labbé et al., 1998; Zhang et al., 1998; Zhou et al., 1998). In contrast to R-Smads and Smad4, the antagonistic Smads, Smad6 and 7, appear to function by blocking ligand-dependent signaling (reviewed in Heldin et al., 1997).

Phosphorylation of R-Smads by the type I receptor is essential for activating the TGFβ signaling pathway (Heldin et al., 1997; Attisano and Wrana, 1998; Kretzschmar and Massagué, 1998). However, little is known of how Smad interaction with receptors is controlled. Here, we describe the identification of a novel Smad2/Smad3 interacting protein that contains a double zinc finger, or FYVE domain, and which we have called SARA. We show that SARA recruits Smad2 into distinct subcellular domains and that SARA colocalizes and interacts with TGFβ receptors. TGFβ signaling induces dissociation of Smad2 from SARA with concomitant formation of Smad2/Smad4 complexes and nuclear translocation. Moreover, deletion of the FYVE domain in SARA causes mislocalization of Smad2 and inhibits TGFβ-dependent transcriptional responses. Thus, SARA defines a component of TGFβ signaling that functions to recruit Smad2 to the receptor by controlling the subcellular localization of Smad.

Results

Identification of SARA

Smad2 is a critical intracellular mediator of the TGFβ signaling pathway (Heldin et al., 1997; Attisano and Wrana, 1998; Kretzschmar and Massagué, 1998). Thus, to define additional components of this pathway, we initiated a screen to identify Smad2 partners. For this, the MH2 domain of Smad2 was fused to glutathione-S-transferase (GST) that included a kinase recognition site for protein kinase A (PKA). The bacterially expressed fusion protein was labeled to high specific activity using PKA (Chen and Sudol, 1995) and then used to screen a λZAPII expression library prepared from the dorsal blastopore lip of *Xenopus*. This yielded repeated isolates

§To whom correspondence should be addressed (e-mail: jwrana@sickkids.on.ca).

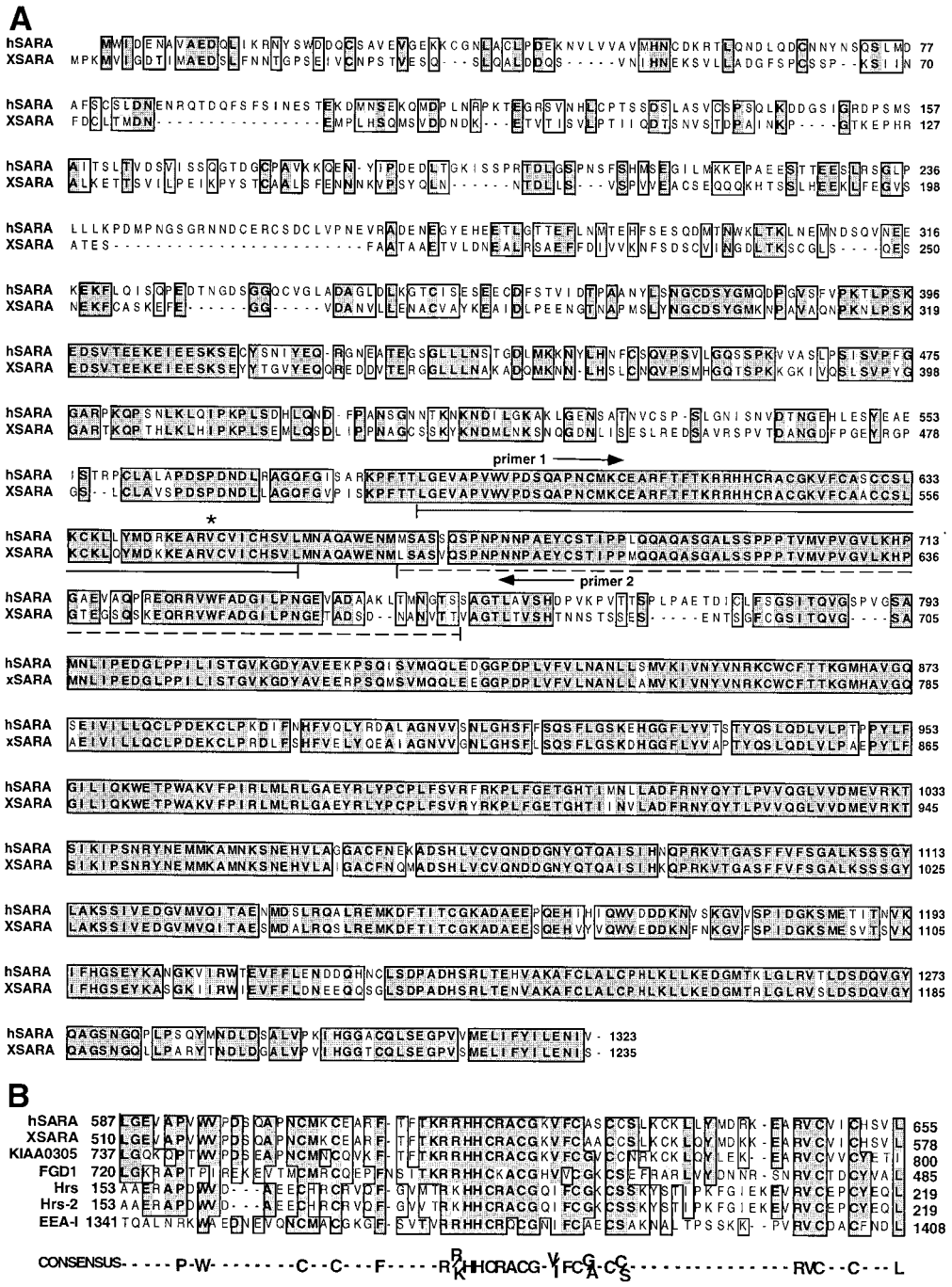

Figure 1. SARA Defines a Class of Conserved Proteins

(A) Comparison of the amino acid sequence of *Xenopus* and human SARA. Identical (dark gray) and conserved residues (light gray), the FYVE domain (solid underline), and the Smad-binding domain (dashed underline) are indicated. Sequences in XSARA used to design degenerate PCR primers for identifying hSARA are shown (arrows). The amino-terminal end of the partial *Xenopus* cDNA obtained in the expression screen is marked (asterisk).

(B) Alignment of the amino acid sequences of the FYVE domains from human and *Xenopus* SARA, KIAA0305, FGD1, Hrs-1, Hrs-2, and EEA1. Identical residues (dark gray) and conservative changes (light gray) are marked. A consensus sequence (bottom) was derived from positions in which at least six out of seven residues were conserved or when proteins contained one of only two alternate residues.

of a partial cDNA clone. To obtain the entire coding sequence, we probed the blastopore lip library with the partial cDNA clone and performed 5′ RACE. Analysis of the complete cDNA sequence revealed a predicted protein of 1235 amino acids with an estimated molecular mass of 135 kDa (Figure 1A). Based on our subsequent functional analysis, we have called this cDNA XSARA, for *Xenopus* Smad anchor for receptor activation.

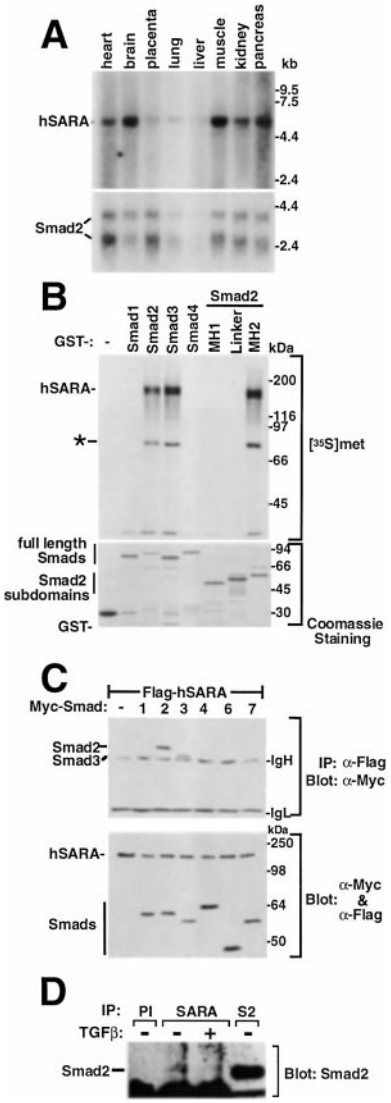

Figure 2. Interaction and Expression Patterns of hSARA and Smad2

(A) hSARA and Smad2 are ubiquitously expressed in similar patterns. An ApaI-SmaI restriction fragment from the 3′UTR of hSARA (top panel) and a Smad2 MH1 domain cDNA fragment (bottom panel) were used to probe a human multiple tissue Northern blot (Clontech). A single transcript of approximately 5.0 kb, corresponding to the full-length hSARA cDNA, is indicated.

(B) In vitro interaction of hSARA with bacterially expressed Smads. Full-length [^{35}S]methionine-labeled SARA produced by in vitro transcription/translation was incubated with sepharose-bound bacterially expressed Smads or Smad2 subdomains, and bound material was visualized by SDS-PAGE and autoradiography. Migration of full-length hSARA and a translation product that initiates from an internal methionine (asterisk) are indicated. GST fusion protein concentrations were determined by Coomassie staining of a protein aliquot (bottom panel).

(C) Interaction of hSARA with Smads in mammalian cells. COS cells were transfected with Flag-tagged hSARA (Flag-SARA) either alone or together with the indicated Myc-tagged Smad constructs. For Smad6, an alternative version lacking the MH1 domain was used (Topper et al., 1997). Cell lysates were subjected to an anti-Flag immunoprecipitation, and coprecipitating Smads were detected by immunoblotting with anti-Myc antibodies. The migration of anti-Flag heavy and light chains (IgH and IgL, respectively) are marked. To confirm efficient expression of hSARA and Smads, aliquots of total cell lysates were immunoblotted with the anti-Flag and anti-Myc antibodies (bottom panel).

(D) Interaction of endogenous SARA and Smad2 in mammalian cells. Lysates from HepG2 cells, either untreated or treated for 30 min with 1 nM TGFβ as indicated, were immunoprecipitated with preimmune sera, affinity-purified anti-SARA antibody, or N19 anti-Smad2/3 antibody. Coprecipitating Smad2 was detected by immunoblotting with a polyclonal anti-Smad2 antibody (Macias-Silva et al., 1998).

To investigate the role of this protein in TGFβ signaling in mammalian cells, we identified a human homolog using a combination of degenerate RT-PCR and screening of a human brain cDNA library. Analysis of the contiguous sequence revealed a long open reading frame that encoded a predicted protein of 1323 amino acids with a consensus start codon preceded by stop codons in all three reading frames. Comparison of this sequence with that from XSARA (Figure 1A) revealed an overall identity of 62% with a divergent 558-residue amino terminal domain (35% identity) followed by a closely related carboxy-terminal domain (85% identity). Given this sequence similarity and subsequent functional analysis, we designate this clone hSARA, for human SARA.

Sequence analysis of SARA (Figure 1A) revealed a region in the middle portion of the predicted protein that had similarity to a double zinc finger domain or FYVE domain. The FYVE domain has been identified in a number of unrelated signaling molecules that include FGD1, a putative guanine exchange factor for Rho/Rac that is mutated in faciogenital dysplasia; the HGF receptor substrate Hrs-1 and its homolog, Hrs-2; EEA1, a protein involved in formation of the early endosome; and the yeast proteins FAB1, VPS27, and VAC1 (reviewed in Wiedemann and Cockcroft, 1998). Recently, analysis of a number of FYVE domains from yeast and mammals has revealed that this motif binds phosphatidyl inositol-3-phosphate (PtdIns(3)P) with high specificity and thus represents a novel signaling module that can mediate protein interaction with membranes (Burd and Emr, 1998; Gaullier et al., 1998; Patki et al., 1998; Simonsen et al., 1998; Wiedemann and Cockcroft, 1998). Comparison of the FYVE domains from the vertebrate proteins with that from SARA revealed extensive conservation of residues throughout the domain (Figure 1B). Thus, SARA contains a FYVE domain that may function to bind PtdIns(3)P.

Analysis of the expression pattern of hSARA revealed that the gene was expressed in all adult tissues examined, similar to Smad2 (Figure 2A). Further analysis in a variety of cell lines by RT-PCR revealed that SARA was expressed in every cell line tested, including HepG2 hepatoma cells, NBFL neuroblastoma cells, SW480 colorectal cancer cells, NIH 3T3 fibroblasts, P19 embryonic carcinoma cells, MC3T3 calvarial cells, and Mv1Lu lung epithelial cells (data not shown). Thus, we conclude that SARA is a ubiquitously expressed partner for Smad2.

hSARA Interacts Specifically with Smad2 and Smad3

To characterize the interaction of hSARA with Smads, we translated the full-length protein in vitro and tested for binding to bacterially expressed Smad fusion proteins. hSARA bound specifically to full-length Smad2

and the highly related Smad3, but not Smad1 or Smad4 (Figure 2B). To define the domains of Smad2 that bound hSARA, various fragments of Smad2 corresponding to the MH1 domain, linker region, and MH2 domain were expressed in bacteria. hSARA interacted efficiently with fusion proteins that comprised the MH2 domain, while no association was detected between hSARA and either the MH1 or nonconserved linker domains (Figure 2B). Similar findings were obtained with *Xenopus* SARA (data not shown). Thus, SARA interacts with Smad2 through the MH2 domain.

To confirm that hSARA also bound to Smads in mammalian cells, we expressed a Flag epitope–tagged version of SARA in COS-1 cells together with Myc-tagged versions of Smads 1, 2, 3, 4, 6, and 7. Cell lysates were subjected to anti-Flag immunoprecipitation followed by immunoblotting with anti-Myc antibodies. In immunoprecipitates of cells expressing either Smad2 or Smad3, efficient coprecipitation of either Smad with Flag-SARA was observed (Figure 2C). In contrast, none of the other Smads coprecipitated with SARA. Specific binding of SARA to both Smad2 and Smad3 is consistent with the observation that these two proteins possess very closely related MH2 domains (97% identity) and are both activated by TGFβ or activin type I receptors (Macías-Silva et al., 1996; Liu et al., 1997b; Nakao et al., 1997).

We also tested for interaction between endogenous SARA and Smad2. For this, SARA was immunoprecipitated from HepG2 cells using an affinity-purified anti-SARA polyclonal antibody, and Smad2 was visualized by immunoblotting with anti-Smad2 antibody (Macías-Silva et al., 1998). In immunoprecipitates prepared with preimmune antisera, no Smad2 was detectable (Figure 2D). However, in the anti-SARA immunoprecipitates, we could clearly detect Smad2 coprecipitating with SARA. Interestingly, TGFβ treatment prior to lysis revealed decreased association of Smad2 with SARA. Together, these results demonstrate that SARA is a specific partner for receptor-regulated Smads in the TGFβ/activin signaling pathway and further suggest that TGFβ signaling induces dissociation of SARA/Smad2 complexes.

Phosphorylation of Smad2 Induces Dissociation from SARA

Activation of TGFβ signaling results in phosphorylation of Smad2 or Smad3 by type I receptors on C-terminal serine residues (Macías-Silva et al., 1996; Liu et al., 1997b; Souchelnytskyi et al., 1997). Thus, we investigated whether receptor-mediated phosphorylation of Smad2 or SARA might regulate their interaction. For this, we used a constitutively active TGFβ type I receptor that regulates phosphorylation and activation of Smad proteins in a manner similar to ligand (Hoodless et al., 1996; Macías-Silva et al., 1996). COS cells transfected with combinations of Smad2, SARA, and receptor were metabolically labeled with [^{32}P]phosphate, and phosphorylation of either SARA or Smad2 was assessed in immunoprecipitates. Analysis of SARA revealed that coexpression of the activated type I receptor did not appreciably affect the overall phosphorylation (Figure 3A). In contrast, the activated type I receptor induced strong phosphorylation of Smad2 as described previously (Macías-Silva et al., 1996). Interestingly, unlike

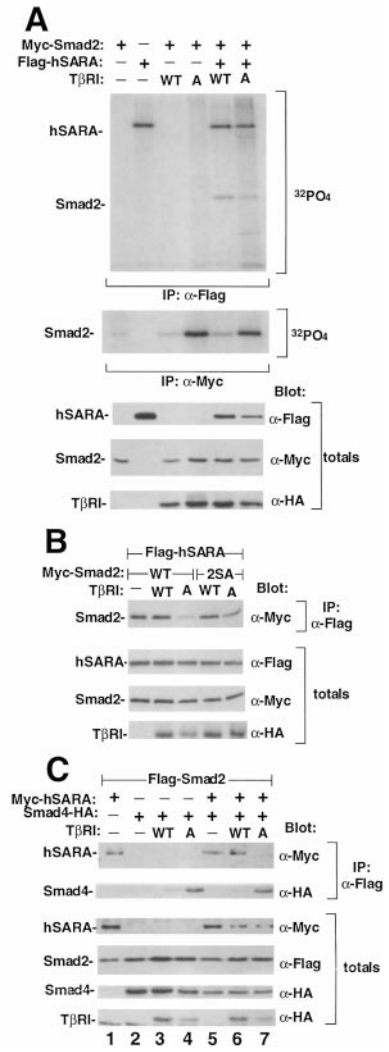

Figure 3. Effects of TGFβ Signaling on SARA and Formation of SARA/Smad2 Complexes

COS cells were transiently transfected with various combinations of Flag or Myc-tagged hSARA; wild-type (WT) or mutant (2SA) Myc or Flag-tagged Smad2; Smad4/HA; and wild type (WT) or constitutively active (A) TβRI/HA. Cell lysates were then subjected to immunoprecipitation with anti-Flag or anti-Myc antibodies, as indicated. Confirmation of protein expression was performed by immunoblotting total cell lysates prepared in parallel for the indicated tagged protein (totals, bottom panels).

(A) Phosphorylation of SARA is not regulated by TGFβ signaling. Transfected cells were labeled with [^{32}P]PO_4, and lysates were subjected to immunoprecipitation with anti-Flag antibodies for visualization of hSARA phosphorylation (top panel) or with anti-Myc antibodies for Smad2 phosphorylation (middle panel).

(B) Activation of TGFβ signaling induces dissociation of SARA/Smad2 complexes. Lysates from transiently transfected cells were subjected to immunoprecipitation with anti-Flag antibodies, and Smad2 bound to SARA was analyzed by immunoblotting with anti-Myc antibodies (IP, α-flag; blot, α-Myc).

(C) SARA/Smad2 and Smad2/Smad4 complexes are mutually exclusive. Cell lysates from transiently transfected cells were subjected to immunoprecipitation with anti-Flag antibodies directed toward Smad2 and then immunoblotted using anti-Myc or anti-HA antibodies that recognize hSARA (α-myc blot) or Smad4 (α-HA blot), respectively.

the strong induction of Smad2 phosphorylation in the total cellular pool, phosphorylation of Smad2 associated with SARA appeared to decrease in the presence of TGFβ signaling (Figure 3A). This suggested that receptor-dependent phosphorylation of Smad2 might induce dissociation from SARA. To examine this directly, we analyzed SARA interaction with wild-type Smad2 or a mutant version lacking the C-terminal phosphorylation sites (Smad2(2SA)). In the absence of TGFβ signaling, association of SARA with either Smad2 or Smad2(2SA) was comparable (Figure 3B). In contrast, in cells coexpressing the activated receptor, we observed a significant decrease in the interaction of wild-type Smad2 with SARA, while SARA/Smad2(2SA) complexes were not reduced. Together, these results suggest that SARA is not phosphorylated in response to TGFβ signaling and that it preferentially interacts with the unphosphorylated form of Smad2.

SARA and Smad4 Form Mutually Exclusive Complexes with Smad2

Phosphorylation of Smad2 induces its interaction with Smad4 (Lagna et al., 1996; Zhang et al., 1997). Since Smad2 also interacts with SARA, we examined the formation of Smad2/Smad4 and Smad2/SARA complexes in the same transfectants in response to TGFβ signaling. For this, lysates from transiently transfected COS cells were subjected to immunoprecipitation with anti-Flag antibodies directed toward tagged Smad2 followed by immunoblotting for the presence of Smad4 and SARA. Consistent with previous findings (Lagna et al., 1996; Zhang et al., 1997), interaction of Smad4 with Smad2 was strongly stimulated by the activated type I receptor (Figure 3C, lane 3 and 4). Concomitant with this, the interaction of Smad2 with SARA was disrupted (Figure 3C, lanes 6 and 7). Furthermore, we never observed association of SARA and Smad4 in the presence of TGFβ signaling (data not shown). Thus, complexes of Smad2/SARA and Smad2/Smad4 are mutually exclusive. Together these results demonstrate that during TGFβ signaling, phosphorylation of Smad2 induces its dissociation from SARA and promotes formation of heteromeric complexes with Smad4.

SARA Regulates the Subcellular Localization of Smad2

Our biochemical analyses of SARA/Smad2 interactions suggested that SARA functions upstream in the pathway and might control the subcellular localization of Smad2. To test this we investigated whether coexpression of SARA might alter the localization of Smad2 in the TGFβ-responsive epithelial cell line, Mv1Lu, using confocal microscopy. In cells expressing SARA alone, the protein displayed a punctate staining pattern that was present throughout the cytosolic compartment and was excluded from the nucleus (Figure 4A). This localization of SARA was in contrast to the diffuse staining typically observed for Smad2 in cells overexpressing the protein (Figure 4B). Since excess Smad2 expression might overwhelm endogenous SARA protein, we next examined cells transiently transfected with both SARA and Smad2. In these cells, the distribution of SARA was indistinguishable from cells transfected with SARA alone (Figure 4D, panel i). In contrast, the localization of Smad2 in the presence of SARA displayed a dramatic shift to a punctate pattern (compare Figure 4B to 4D, panel ii). Moreover, analysis of these immunofluorescent staining patterns by confocal microscopy revealed that SARA and Smad2 precisely colocalized in the cytosol (yellow stain, Figure 4D, panel iii). Interestingly, expression of Smad2 at much higher levels than SARA reverted the distribution of Smad2 to that observed in cells transfected with Smad2 alone (data not shown). This supports the notion that elevating the amount of Smad2 can saturate SARA and yield a diffuse distribution of Smad2 throughout the cell.

Since phosphorylation of Smad2 results in dissociation from SARA, we investigated whether activation of TGFβ signaling induces nuclear translocation of Smad2 in the presence of SARA. As shown in Figure 4, the localization of SARA in the cytosolic compartment looked similar in the presence or absence of the constitutively active TGFβ type I receptor (compare Figures 4D and 4E, panels i). However, TGFβ signaling caused a significant proportion of Smad2 to translocate to the nucleus (Figure 4E, panel ii), and this correlated with a shift to an orange-red color in the cytosolic colocalization stain (Figure 4E, panel iii). Thus, activation of TGFβ signaling induces Smad2 to dissociate from SARA and translocate to the nucleus.

To confirm that the punctate localization of overexpressed SARA reflected that of the endogenous protein, we examined SARA and Smad2 in Mv1Lu cells. Analysis of the distribution of endogenous SARA using rabbit anti-SARA antibodies revealed a punctate distribution similar to that observed for transiently transfected, epitope-tagged SARA (Figure 4F, panel i). This staining was specific, since cells stained with preimmune antisera or purified antibody blocked with the SARA antigen revealed no detectable staining in the cytosol (data not shown). Examination of endogenous Smad2 distribution in the same cell revealed a punctate distribution for Smad2 (Figure 4F, panel ii) as published previously (Janknecht et al., 1998). Furthermore, analysis of SARA and Smad2 together revealed extensive colocalization of the two proteins (Figure 4F, panel iii). Colocalization was not complete and may reflect differences in the stoichiometry of SARA versus Smad2 protein levels, as suggested above, or the presence of additional regulatory mechanisms that control interaction of the endogenous proteins. Taken together with our biochemical analysis, these results indicate that SARA functions to recruit Smad2 to specific subcellular regions in the cell prior to activation by TGFβ signaling.

SARA Colocalizes with TβRII

The positioning of SARA upstream of Smad2 activation suggested to us that SARA might recruit Smad2 to specific subcellular domains for phosphorylation and activation by the receptor. Interestingly, previous studies on the TGFβ receptor demonstrated clustering of the receptor complex into punctate domains that resembled those displayed by SARA (Henis et al., 1994; Gilboa et

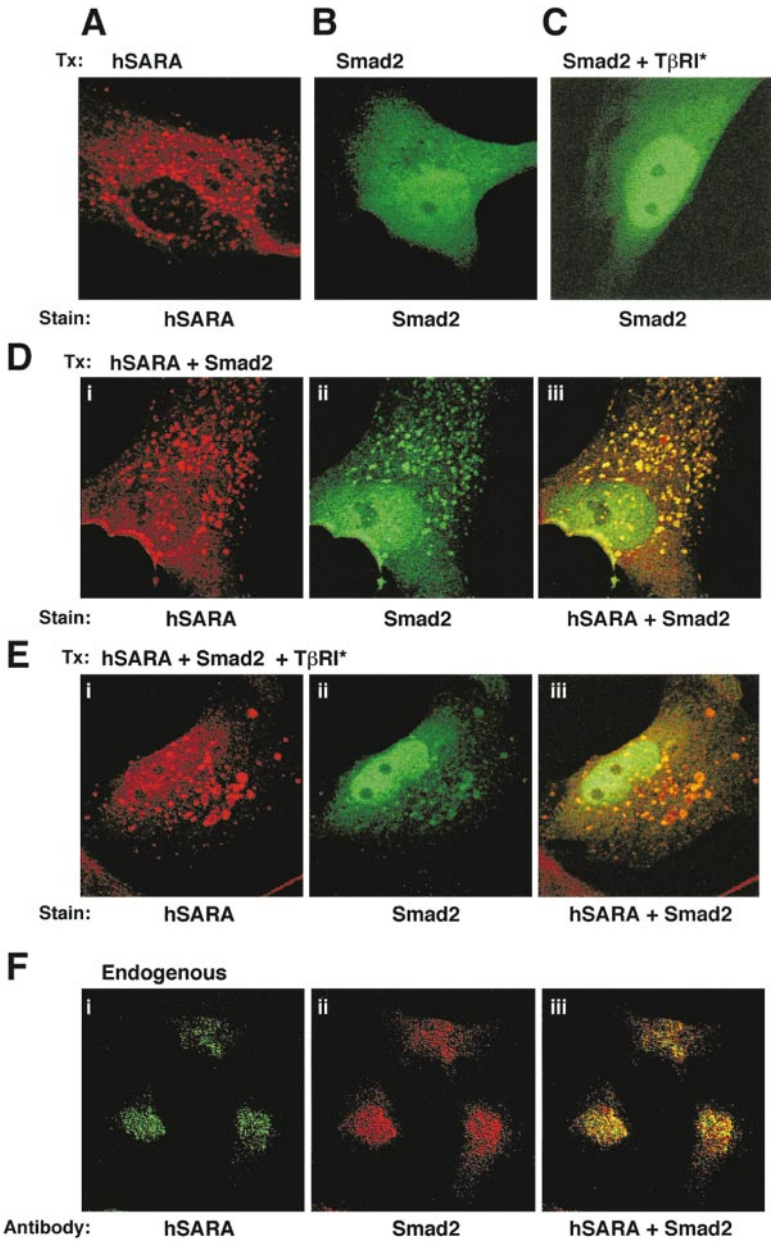

Figure 4. SARA Regulates the Localization of Smad2

(A–E) Mv1Lu cells were transiently transfected with hSARA alone (A), Smad2 alone (B), Smad2 with constitutively active TβRI (TβRI*, [C]), or Flag-Smad2 with Myc-hSARA with (E) or without (D) constitutively active TβRI as indicated (Tx). hSARA was visualized with the polyclonal Myc A14 antibody, and Texas red–conjugated goat anti-rabbit IgG (red) and Smad2 was detected with an anti-Flag M2 monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG (green). The subcellular localization of the expressed proteins was visualized by immunofluorescence and confocal microscopy. Colocalization of SARA and Smad2 appears as yellow. (F) Colocalization of endogenous SARA and Smad2/3. Endogenous SARA in Mv1Lu cells was visualized with polyclonal rabbit anti-SARA antibody (panel i, green), and Smad2/3 was visualized with the polyclonal goat anti-Smad2/3 N19 antibody (Santa Cruz) (panel ii, red). Colocalization of SARA and Smad2 is shown (panel iii) and appears as yellow.

al., 1998). Thus, to test whether SARA might colocalize with TGFβ receptors, Mv1Lu cells transfected with SARA and TGFβ receptors were treated with TGFβ, and protein subcellular localization was determined. As observed previously (Henis et al., 1994; Gilboa et al., 1998), TβRII displayed a punctate staining pattern similar to the SARA pattern (Figure 5A, panels i and ii). Further, in cells coexpressing SARA and TGFβ receptors, extensive colocalization of the proteins was observed (Figure 5A, panel iii). This colocalization was not complete, possibly reflecting a restricted distribution of SARA to only a subset of the intracellular compartments normally occupied by transmembrane receptors, which include the endoplasmic reticulum, Golgi, and endocytic compartments. Thus, SARA and the TGFβ receptors colocalize to common subcellular domains.

Since SARA and TGFβ receptors colocalize, we tested whether SARA might interact with the receptors. As described previously for Smad2 (Macías-Silva et al., 1996), COS cells were cotransfected with TGFβ receptors in the presence of SARA and then affinity labeled using [^{125}I]TGFβ. SARA was immunoprecipitated from the cell lysates, and coprecipitating receptor complexes were visualized by autoradiography or quantitated using a gamma counter. Analysis of cells expressing wild-type receptors revealed that these complexes coprecipitated with SARA (Figure 5B, lane 3). Furthermore, in the presence of kinase-deficient type I receptor, there was a small increase in binding of SARA to the receptor (Figure 5B, lane 2). This is in contrast to Smad2 that only interacts with TGFβ receptor complexes that contain kinase-deficient type I receptors (Macías-Silva et al., 1996). These data suggest that SARA associates with the TGFβ receptor.

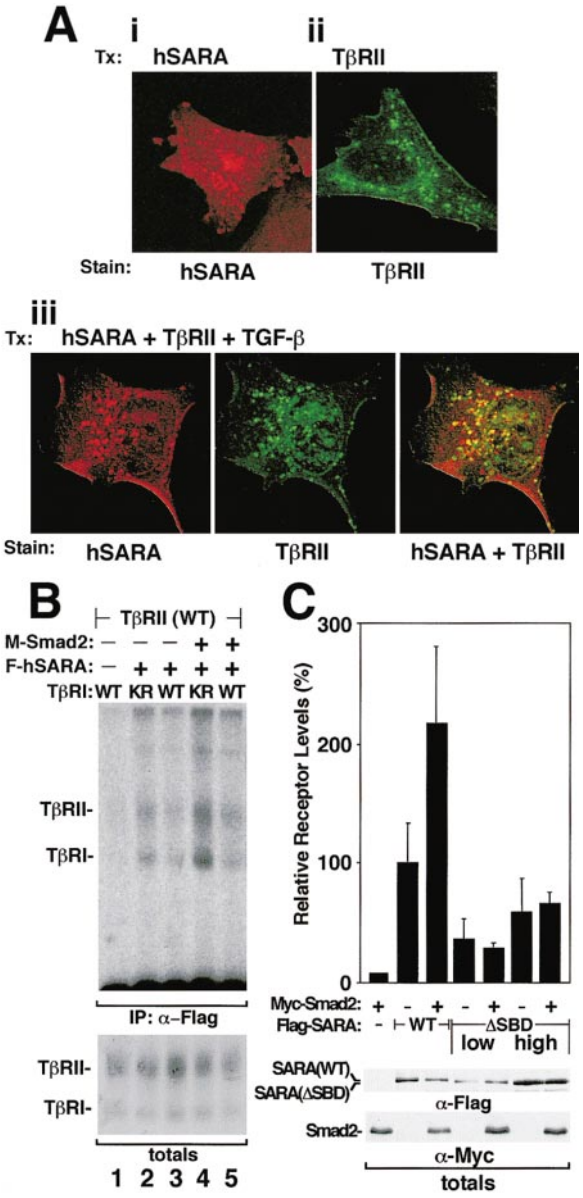

Figure 5. SARA Interacts with Receptor Complexes

(A) Mv1Lu cells were transfected with either hSARA alone (panel i), TβRII alone (panel ii), or SARA and TβRII together (panel iii). Cells were then treated with TGFβ, and the localization of hSARA (red) and TβRII (green) was determined by immunofluorescence and confocal microscopy. Colocalization of SARA and TβRII appears as yellow (panel iii).

(B) SARA interacts with the TGFβ receptor. COS cells were transiently transfected with various combinations of Flag-hSARA, Myc-Smad2, wild type (WT) TβRII, and either wild-type or kinase-deficient (KR) versions of TβRI. Cells were affinity labeled with [^{125}I]TGFβ and lysates immunoprecipitated with anti-Flag antibodies. Coprecipitating receptor complexes were visualized by SDS-PAGE and autoradiography. Equivalent receptor expression was confirmed by visualizing aliquots of total cell lysates (bottom panel).

(C) Smad2 binding to SARA enhances receptor interaction. COS cells were transiently transfected with wild-type TβRII and kinase-deficient TβRI and various combinations of wild-type Flag-hSARA (WT), a mutant version lacking the Smad2-binding domain (ΔSBD) and Myc-Smad2. The amount of receptor bound to SARA was determined by anti-Flag immunoprecipitation followed by gamma counting. Data are plotted as the average of three experiments ± SD. Protein expression was analyzed by immunoblotting aliquots of total cell lysates, and the results from a representative experiment are shown (bottom panel).

We next examined whether coexpression of Smad2 might enhance the interaction of SARA with TGFβ receptors. In cells expressing wild-type receptor I, we observed no difference in the amount of receptor complexes that coprecipitated with SARA either in the presence or absence of Smad2 (Figure 5B, compare lanes 3 and 5). In contrast, the association of SARA with receptor complexes containing kinase-deficient type I receptors was enhanced by Smad2 (Figure 5B, lane 4). This finding was consistent with our previous demonstration that kinase-deficient type I receptors stabilize interactions of Smad2 with the receptors (Macías-Silva et al., 1996). To investigate further the requirement for Smad2 in the interaction of SARA with the receptor, we also tested a mutant of SARA, SARA(ΔSBD), that removes the Smad-binding domain (see below). Analysis of SARA interaction with receptor complexes containing kinase-deficient TβRI showed that wild-type SARA interacted with the receptor, and this was enhanced approximately 2-fold by Smad2 (Figure 5C). The ΔSBD mutant of SARA retained the capacity to associate with the receptor, although the efficiency of interaction was slightly reduced relative to wild-type SARA. Importantly, unlike wild-type SARA, binding of mutant SARA to the receptor was not enhanced by coexpression of Smad2. Together, these data suggest that SARA interacts with the TGFβ receptor independently of Smad2 binding and that Smad2 cooperates to enhance association.

A Modular Domain in SARA Mediates Association with Smads

To investigate the functional importance of SARA in TGFβ signaling, we defined the domains in the protein that mediate its interaction with Smad2, its localization to specific subcellular regions, and its association with the TGFβ receptor. To this end, we constructed a series of deletion mutants of SARA and first tested their ability to interact with Smad2 in COS cells by immunoprecipitation followed by immunoblotting. As summarized in Figure 6A, loss of the first 664 amino acids of SARA, which included the double zinc finger/FYVE domain, did not interfere with SARA binding to Smad2, while further deletions (Δ1–704) completely abolished the interaction. A similar analysis of C-terminal truncations revealed that deletion of residues upstream of position 749 (Δ665–1323) completely abrogated binding to Smad2. To determine whether the region defined by this deletional analysis was sufficient to bind Smad2, we linked the 85 amino acids we refer to as the Smad-binding domain (SBD) to GST and expressed the fusion protein in bacteria (GST-hSARA[665–750]). Incubation of lysates prepared from cells expressing Smad2 or Smad3 with GST-SBD resulted in efficient binding of both Smads to the fusion protein (Figure 6B). This interaction is likely direct, since bacterially expressed SBD associates efficiently with bacterially produced Smad2 (data not shown). These studies thus define a novel domain in SARA that mediates interaction with Smad2 and Smad3 and which is located downstream of the FYVE domain.

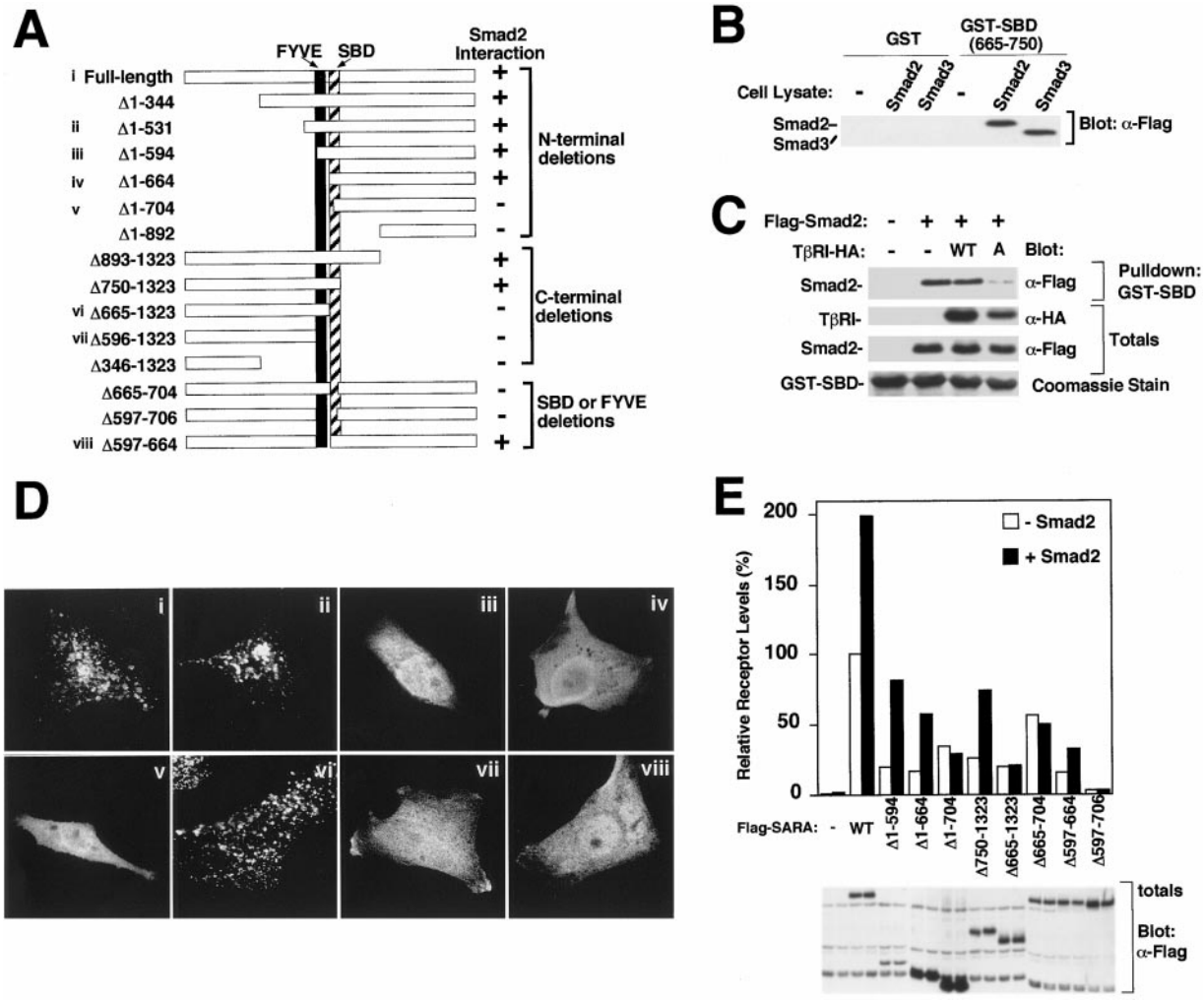

Figure 6. Mapping the Functional Domains on SARA

(A) Schematic representation of mutant versions of SARA. The FYVE domain (black bar) and the Smad-binding domain (SBD; striped bar) are indicated. COS cells transiently transfected with Flag-hSARA and Myc-Smad2 were immunoprecipitated with anti-Flag antibodies followed by immunoblotting with anti-Myc antibodies. The presence (+) or absence (−) of a SARA/Smad2 interaction is indicated (Smad2 interaction). Mutants used for the subsequent localization study are marked on the left (i–viii).

(B) The Smad-binding domain (SBD) of SARA is sufficient for interaction with Smad2 and Smad3. Lysates from COS cells expressing Flag-tagged Smad2 or Smad3 were incubated with GST alone or with GST-hSARA (665–750), which corresponds to the SBD, and bound proteins were immunoblotted using anti-Flag antibodies. The presence of Smad2 and Smad3 bound to GST-hSARA(665–750) is indicated.

(C) The Smad-binding domain (SBD) of SARA binds preferentially to unphosphorylated Smad2. Lysates from COS cells expressing Flag-tagged Smad2 together with wild-type (WT) or activated (A) type I receptor were incubated with GST-hSARA(665–750) (GST-SBD), and bound Smad2 was immunoblotted using anti-Flag antibodies. Note that Smad2 from cell lysates cotransfected with the activated type I receptor display reduced interaction with GST-SBD. The expression levels of Smad2, each receptor, and GST-hSARA(665–750) are indicated.

(D) Subcellular localization of SARA mutants. Mv1Lu cells were transiently transfected with wild-type (panel i) or mutant versions of Flag-hSARA (panels ii–viii, as marked on the left in [A]). Proteins were visualized by immunofluorescence and confocal microscopy using a monoclonal anti-Flag M2 monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG.

(E) Identification of receptor-binding domain of SARA. COS cells were transiently transfected with wild-type TβRII and kinase-deficient TβRI and Flag-tagged wild-type (WT) or mutant versions of SARA with (black bars) or without (open bars) Myc-Smad2. The amount of receptor bound to SARA was determined by anti-Flag immunoprecipitation followed by gamma counting (as in Figure 5). Protein expression was analyzed by immunoblotting aliquots of total cell lysates (bottom panel).

Our previous analysis showed that activation of Smad2 by the TGFβ receptor induced dissociation from SARA. To determine whether this reflects an alteration in the ability of the SBD to bind phosphorylated Smad2, we tested the interaction of GST-SBD with Smad2 in lysates obtained from cells expressing Smad2 alone or together with either wild-type or activated TGFβ type I receptor. Consistent with our previous observations, phosphorylation of Smad2 by activated TβRI strongly reduced interactions with GST-SBD (Figure 6C). This correlated with receptor-dependent phosphorylation, since the phosphorylation site mutant, Smad2(2SA), interacted efficiently with GST-SBD, even in the presence of activated TβRI (data not shown). Together with our previous

results, these data strongly support the notion that SARA interacts with unphosphorylated Smad2 and that receptor-dependent phosphorylation induces dissociation by altering the affinity of Smad2 for the SBD.

The FYVE Domain Controls the Subcellular Localization of SARA

We next analyzed the subcellular localization of a selection of our SARA mutants by immunofluorescence and confocal microscopy. Analysis of truncation mutants that removed the amino terminus upstream of the FYVE domain (Δ1–531) yielded wild-type patterns of staining (Figure 6D, compare panels i and ii). However, further deletions that remove the conserved amino-terminal portion (Δ1–594) or the entire FYVE domain (Δ1–664, Δ1–704) abolished the wild-type staining pattern (Figure 6D, panels iii–v) even in the presence of an intact Smad-binding domain (Δ1–594, Δ1–664; Figure 6D, panels iii and iv). Similar studies of the C-terminal domain mutants showed that deletion of residues downstream of the FYVE domain (Δ665–1323) did not alter the localization of the mutant protein (Figure 6D, panel vi), while truncations to within the FYVE domain (Δ596–1323) led to diffuse localization throughout the cell (Figure 6D, panel vii). Of note, the Δ665–1323 mutant that lacks the Smad-binding domain had a wild-type staining pattern, thereby indicating that interaction with Smad2 is not required for proper SARA localization. To confirm that FYVE domain function was required for localization of SARA, we also tested a mutant with a small internal deletion that removes the FYVE domain (Δ597–664). Consistent with our other mutants, localization of this protein was clearly disrupted (Figure 6D, panel viii). Thus, we conclude that the FYVE domain is required to maintain the normal localization of SARA but is not involved in mediating interaction with Smads.

The C-Terminal Domain of SARA Interacts with the TGFβ Receptor

To characterize the domains in SARA that mediate binding to the TGFβ receptor, we tested the interaction of our panel of SARA mutants with the TGFβ receptor. Interestingly, interaction with the TGFβ receptor was strongly suppressed but not abolished in three mutants in which the FYVE domain was disrupted (Figure 6E; Δ1–594, Δ1–664, and the internal deletion Δ597–664). Since the FYVE domain is required for the correct subcellular localization of SARA, we reasoned that once bound to the membrane, other regions in SARA might contribute to the interaction with the receptor. To examine this possibility, we tested several carboxy-terminal truncation mutants. Interestingly, deletion of the C terminus downstream of position 750 suppressed receptor interaction, despite efficient expression of the truncated protein. This suggests that regions in the carboxyl terminus of SARA contribute to receptor interaction. In these analyses we also explored whether overexpression of Smad2 could rescue interaction of SARA mutants with the receptor. For both the FYVE domain mutants and the C-terminal truncation, Smad2 expression was able to restore some interaction with the TGFβ receptor. It is likely that the high levels of protein and receptor expression that are achieved in COS cells can drive some receptor interaction, even in the absence of appropriate localization signals.

Together, these data define discrete domains in SARA that fulfill specific aspects of SARA function in TGFβ signaling. The FYVE domain likely functions to direct SARA to the membrane and by analogy with other FYVE domain proteins, may do so through interactions with PtIns(3)P (Wiedemann and Cockcroft, 1998). It thus fulfills an important role in recruiting SARA to specific subcellular domains that we show also contain the TGFβ receptor. The SBD in turn functions to bind unactivated Smad2, thus recruiting the receptor substrate to this subcellular region. Once localized to this region, the C-terminal domain of SARA functions with Smad2 bound to the SBD to promote interaction with the receptor complex. These three domains thus function cooperatively to recruit Smad2 to the TGFβ receptor.

SARA-Mediated Localization of Smad2 Is Necessary for TGFβ Signaling

The availability of mutants of SARA that interact with Smad2 but fail to target to the appropriate subcellular sites allowed us to address the question of whether SARA-mediated localization of Smad2 was important in TGFβ signaling. We first tested whether SARA (Δ1–594) and SARA (Δ1–664), which bind Smad but fail to distribute to the correct subcellular domains, would mislocalize Smad2. Coexpression of either mutant with Smad2 showed that they were unable to recruit Smad2 to the normal SARA domains (Figure 7A, panel i and ii). As expected, SARA(Δ1–704), which lacks a Smad-binding domain, was unable to control Smad2 localization (Figure 7A, panel iii). We also examined directly whether these mutants could cause mislocalization of Smad2. For this, cells were cotransfected with wild-type SARA and Smad2 either in the absence or presence of SARA(Δ1–594), SARA(Δ1–664), or SARA(Δ1–704). In control transfectants performed in the absence of mutant SARA, SARA and Smad2 were colocalized in punctate domains as described above (Figure 7B, panel i). However, in the presence of either SARA(Δ1–594) or SARA(Δ1–664), the localization of wild-type SARA was normal, but the distribution of Smad2 was clearly disrupted and displayed a diffuse pattern (Figure 7B, panels ii and iii, respectively). Moreover, coexpression of SARA(Δ1–704), which does not bind Smad2, resulted in Smad2 distribution that was indistinguishable from that of the wild-type pattern (Figure 7B, panel iv). Thus, SARA(Δ1–594) and SARA(Δ1–664) induce the mislocalization of Smad2.

Since SARA(Δ1–664) mislocalizes Smads and interferes with receptor association, we investigated whether this mutant would disrupt TGFβ signaling. To test this, we transiently transfected the TGFβ-responsive reporter gene 3TP-lux into Mv1Lu cells in the presence and absence of wild-type or mutant versions of SARA. Expression of wild-type SARA had no effect on TGFβ signaling (Figure 7C). In contrast, transfection of SARA(Δ1–664) significantly inhibited TGFβ-dependent signaling at the lowest concentration of DNA tested, while transfection of higher doses completely abolished responsiveness of the cells. We also tested SARA(Δ1–704), which lacks a functional Smad-binding domain and does not alter Smad2 localization. Transfection of this mutant had no

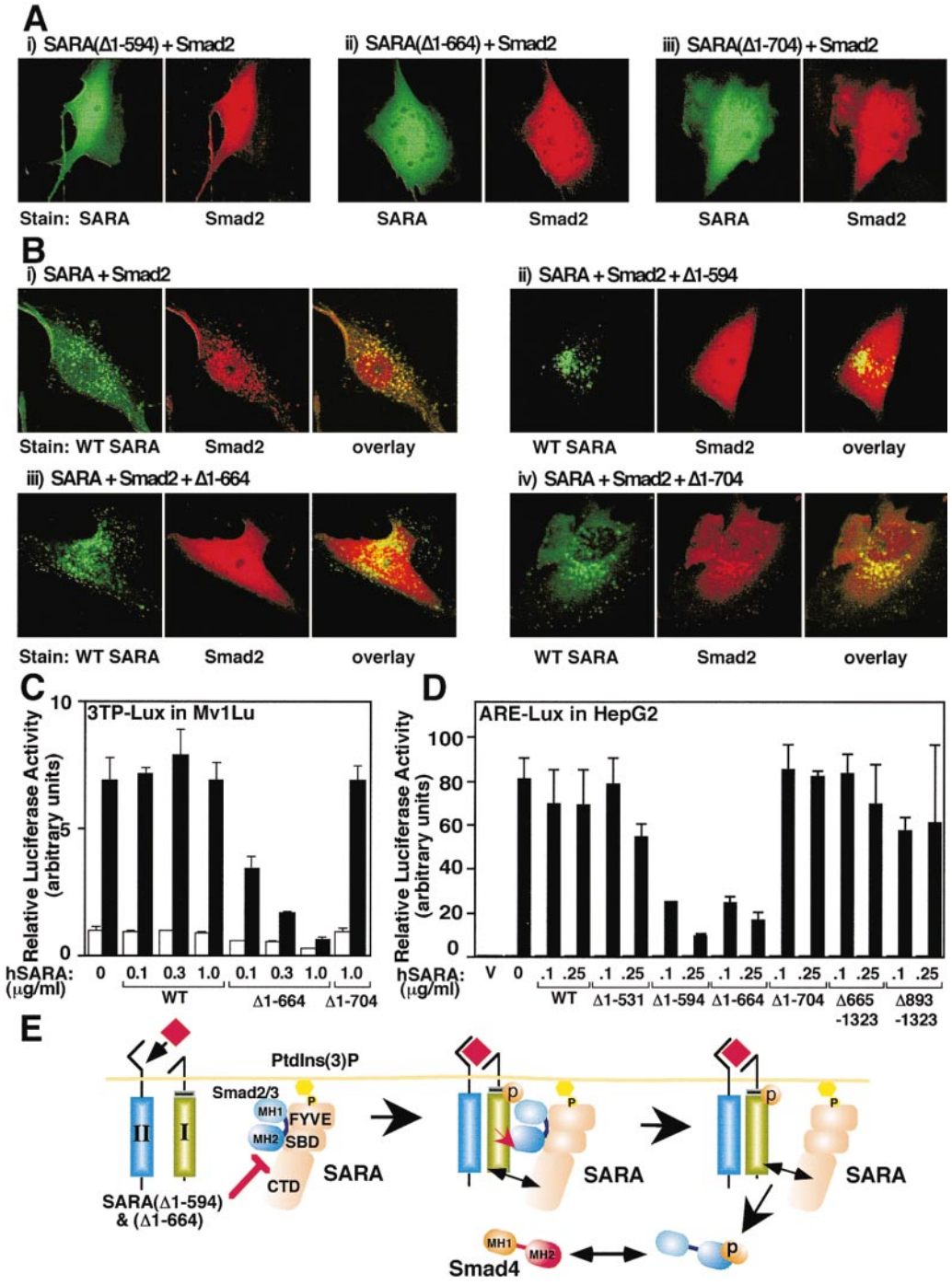

Figure 7. Mislocalization of Smad2 by SARA Mutants Blocks TGFβ Signaling

(A and B) SARA mutants lacking the FYVE domain mislocalize Smad2. Mv1Lu cells were transiently transfected with mutant versions of Myc-hSARA and Flag-Smad2 (A) or with wild-type Myc-hSARA, HA-Smad2, and mutant versions of hSARA (B) as indicated. Protein subcellular localization was visualized by immunofluorescence and confocal microscopy. hSARA was visualized with the polyclonal Myc A14 antibody and FITC-conjugated goat anti-rabbit IgG (green), while Smad2 was detected with monoclonal antibodies followed by Texas red–conjugated goat anti-mouse IgG (red). In (B), overlaying the images reveals mislocalization of Smad2 as green speckles of SARA over red, diffuse Smad2 staining (panels ii and iii), and colocalization of SARA and Smad2 appears as yellow spots (panels i and iv).

(C and D) FYVE domain mutants of SARA block TGFβ-dependent activation of transcription. (C) Mv1Lu cells were transfected with 3TP-lux alone or together with the indicated amounts of wild-type (WT) or mutant (Δ1–664 or Δ1–704) versions of hSARA. (D) HepG2 cells were transfected with ARE-Lux alone (v), or ARE-Lux and FAST2 alone or together with the indicated amounts of wild-type (WT) or mutant versions of SARA. Transfected cells were incubated in the presence (black bars) or absence (open bars) of TGFβ, and luciferase activity was normalized to β-galactosidase activity and is plotted as the mean ± SD of triplicates from a representative experiment.

(E) A model for SARA function in TGFβ signaling. See discussion for details.

effect on TGFβ signaling (Figure 7C). In addition to analysis of the 3TP promoter, we examined induction of the activin response element (ARE) from the *Xenopus Mix.2* gene in HepG2 cells. This ARE is stimulated by either TGFβ or activin signaling, which induces assembly of a DNA-binding complex that is composed of Smad2, Smad4, and a member of the FAST family of forkhead DNA-binding proteins. Since HepG2 cells do not possess endogenous FAST activity, we cotransfected wild-type or mutants of SARA, together with FAST2 and the ARE-lux reporter plasmid as described previously (Labbé et al., 1998). Expression of either SARA(1-Δ594) or SARA(1-Δ664), which interfere with normal Smad2 localization, resulted in a strong suppression of TGFβ-dependent induction of the ARE (Figure 7D). However, none of the other mutants that we tested suppressed activation of this promoter. Since none of these latter mutants disturb the localization of SARA–Smad2 complexes, these data strongly suggest that recruitment of Smad2 to the receptor containing subcellular domains is important for TGFβ signaling.

Discussion

The regulation of the subcellular localization of components of signaling pathways can be key determinants in the effective initiation and maintenance of signaling cascades. Targeting the location of signal transduction pathways through protein–protein and protein–lipid interactions can thus facilitate activation of a pathway by localizing kinases with their downstream substrates (Faux and Scott, 1996; Pawson and Scott, 1997; Schaeffer et al., 1998; Whitmarsh et al., 1998). Here, we have identified SARA, a protein that binds directly and specifically to unphosphorylated Smad2 and Smad3. SARA contains a lipid-binding FYVE domain and functions in TGFβ signaling to recruit Smad2 to the TGFβ receptor by mediating the specific subcellular localization of Smad and by associating with the TGFβ receptor complex. Furthermore, inducing mislocalization of Smad2 by expressing a mutant of SARA blocks TGFβ-dependent transcriptional responses, indicating an important role for SARA-mediated localization of Smads in signaling. Together, these results suggest a model (Figure 7E) in which SARA is localized to specific subcellular regions in the cell through interactions between the FYVE domain and PtdIns(3)P. This in turn leads to recruitment of Smad2 through interactions with the SBD. Once TGFβ signaling is initiated, SARA and Smad2 cooperatively interact with the receptor complex. Upon phosphorylation, Smad2 dissociates, binds to Smad4, and translocates to the nucleus, freeing SARA for further recruitment of Smads.

Smad Recruitment in TGFβ Signaling

The colocalization and association of SARA with the TGFβ receptor defines a role for SARA in recruiting Smad2 to the receptor kinase. Our finding that deletion of the FYVE domain interferes with receptor binding, prevents the correct localization of SARA/Smad2, and blocks TGFβ signaling suggests that this is an important function in the pathway. However, binding of SARA to the receptor occurred cooperatively with Smad2, and on its own SARA may interact inefficiently with the complex. Thus, the primary mechanism for SARA recruitment of Smad2 to the receptor is likely through its ability to regulate Smad2 localization.

What feature of the TGFβ pathway might define a requirement for recruiting receptor-regulated Smads to specific subcellular domains? In intact cells, receptor-regulated Smads are cytosolic proteins that require activation by transmembrane serine/threonine kinase receptors. Consequently, recruitment of Smads may be required to facilitate interaction with the TGFβ receptor. Consistent with this, we observed that the domains in which SARA is found correspond to regions where the receptors are also localized. Thus, TGFβ receptors display regionalized localization, and SARA recruits Smad2 to these domains. The identity of these intracellular domains is still under investigation. However, PtdIns(3)P binding by FYVE fingers is conserved in yeast and mammals, so it is likely that the FYVE finger in SARA similarly mediates interaction with the membrane. Furthermore, these domains also contain receptors, so it is reasonable to suggest that they are membrane vesicles. Thus, clustering of the TGFβ receptor, as previously described (Henis et al., 1994; Gilboa et al., 1998), may function to direct the receptor to SARA and the Smad2 substrate. Facilitating interactions between the receptor kinase and its Smad substrate may be most critical in vivo, where ser/thr kinase receptors are often found in low numbers and only a small proportion need to be activated for full biological responses (Dyson and Gurdon, 1998).

SARA as a Control Point for Smad Activation and TGFβ Signaling

We observed that elevating Smad2 levels can saturate SARA and yield a diffuse distribution for Smad2. Thus, the level of SARA protein is a key determinant in controlling Smad2 localization. As a consequence, endogenous Smad2 may or may not display a SARA-like distribution, depending on the relative expression of the two proteins. Indeed, in Mv1Lu cells endogenous Smad2 displays a punctate pattern with some diffuse staining in the cytosol (this study and Janknecht et al., 1998), and we did not observe complete colocalization with SARA. However, once signaling has commenced, Smad2 dissociates from SARA, binds to Smad4, and translocates to the nucleus, freeing SARA to recruit additional Smad2 from the cytosolic reservoir. This would provide a mechanism to allow quantitative activation of Smads in the presence of high levels of TGFβ signaling. By functioning to recruit Smad2 to the TGFβ receptor, SARA is located in an important regulatory position in the pathway. Thus, control of SARA localization, protein levels, or interactions with Smad2 could potentially modulate TGFβ signaling. Further, disruption of normal SARA function could potentially be involved in loss of TGFβ responsiveness that is a common feature during tumor progression. We are currently mapping the chromosomal location of SARA to explore this latter possibility.

SARA Defines a Family of Related Proteins

SARA may define a new class of FYVE domain proteins that function to recruit components of signaling cascades to specific regions of the cell. Indeed, in the

course of this work we identified and cloned two other SARA-like proteins. One of these, from *Xenopus*, is closely related to SARA (T. T. et al., unpublished data), while the second one is a human clone, distantly related to SARA, that was also identified in an EST project (clone KIAA0305). KIAA0305 has a FYVE domain, and comparison of the SBD from SARA with the same region in KIAA0305 reveals that the amino-terminal half is highly divergent. This suggests that in KIAA0305 this domain may anchor other, as yet unidentified proteins, that function either in TGFβ or other signaling pathways.

Additional Roles for SARA

Controlling the localization of kinases and their substrates may allow not only for efficient recognition and phosphorylation but may also function to maintain specificity and suppress cross talk between signaling pathways. Thus, by controlling Smad localization, SARA could additionally function to maintain the highly specific regulation of Smad phosphorylation by ser/thr kinase receptors that is observed in vivo and could prevent promiscuous phosphorylation by other kinases in the cell. Furthermore, through its interactions with the TGFβ receptor, SARA might function to control the activity or turnover of the receptor complex. Alternatively, SARA may also fulfill scaffolding functions to coordinate the receptor-dependent activation of Smads with other as yet unidentified components of the TGFβ signaling pathway.

Experimental Procedures

Isolation of *Xenopus* and Human SARA

For expression library screening, the Smad2 MH2 domain (amino acids 241–467) was subcloned into a modified pGEX4T-1 vector (Pharmacia) containing a protein kinase A recognition site. Bacterially expressed protein was purified, labeled with [^{32}P]γATP, and used as probe to screen a λZAP II *Xenopus* dorsal lip library (kindly provided by C. Wright, Vanderbilt) as described (Chen and Sudol, 1995). A screen of 1×10^6 plaques yielded four repeated phage isolates encoding a 3.1 kb partial cDNA. A full-length clone was obtained by a combination of rescreening of the same dorsal lip library using a 670 base pair EcoRI/HpaI fragment at the 5′ end of this clone and by 5′ RACE (GIBCO BRL) using stage 10 *Xenopus* RNA.

To obtain a human homolog of *Xenopus* SARA, cDNA synthesized from randomly primed HepG2 cell total RNA was subjected to PCR using degenerate primers as described previously (Attisano et al., 1992). The 5′ and 3′ primers, designed to encode the zinc-finger motif correspond to GC(A/C/G/T)CC(A/C/G/T)AA(C/T)TG(C/T)ATGA A(A/C/G/T)TG(C/T) and (A/G)CA(A/G)TA(C/T)TC(A/C/G/T)GC(A/C/G/T)GG(A/G)TT (A/G)TT, respectively. The resulting 150 bp PCR product was then used as probe for screening a λZAP human fetal brain cDNA library (Stratagene). Eight positive plaques were obtained, two of which contained an overlap of approximately 1 kb and covered the entire open reading frame. The sequence of the 5′ UTR was confirmed by sequencing of an expressed sequence tag database clone (clone ID 260739).

Construction of Plasmids

For mammalian expression constructs of SARA, the open reading frame was amplified by PCR and was subcloned into pCMV5 in frame with an amino-terminal Flag or Myc tag (Hoodless et al., 1996). The SARA deletion mutants were constructed by restriction enzyme digestion of internal sites or by PCR. pCMV5B-Myc-Smad3 and Myc-Smad6, pGEX4T-1-Smad2/MH1 (amino acids 1–181), pGEX4T-1-Smad2/linker (amino acids 186–273), pGEX4T-1-Smad2/MH2 (amino acids 241–467), and pGEX4T-1-hSARA (amino acids 665–750) were constructed by PCR.

In Vitro Protein Interactions

In vitro transcription/translation reactions were performed using the TNT coupled reticulocyte lysate system (Promega) following the manufacturer's instructions using T3 RNA polymerase. Translation was carried out in the presence of [^{35}S]methionine, and labeled proteins were incubated with purified GST fusion proteins in TNTE buffer with 10% glycerol for 2 hr at 4°C and then washed five times with the same buffer. Bound protein was separated by SDS-PAGE and visualized by autoradiography.

Immunoprecipitation, Immunoblotting, and Affinity Labeling

COS-1 cells transfected by LipofectAMINE (GIBCO BRL) were lysed with lysis buffer (Wrana et al., 1994) and subjected to immunoprecipitation with either anti-Flag M2 (IBI, Eastern Kodak) or anti-Myc (9E10) monoclonal antibody followed by adsorption to protein G-Sepharose. Precipitates were analyzed by immunoblotting as described previously (Hoodless et al., 1996). For affinity labeling, transfected COS-1 cells were incubated with 200 pM [^{125}I]TGFβ in media containing 0.2% bovine fetal serum at 37°C for 30 min, and receptors were cross-linked to ligand with DSS as described previously (Macías-Silva et al., 1996). Cell lysates were immunoprecipitated with anti-Flag antibody, and receptors were visualized by SDS-PAGE and autoradiography or were quantitated using a gamma counter.

Preparation of Polyclonal Antiserum against hSARA

Rabbit polyclonal antiserum was raised against bacterially produced GST-hSARA (aa 1011–1172). Antisera was purified using an affinity matrix prepared by coupling GST-hSARA to Hi-Trap NHS-activated Sepharose (Amersham Pharmacia) by incubating in 0.2 M $NaHCO_3$, 0.5 M NaCl (pH 8.3) for 2 hr. Antibody was bound to the GST-hSARA matrix, eluted from the column in 100 mM glycine (pH 2.5), neutralized in 1 M Tris (pH 8), and dialyzed overnight in phosphate-buffered saline (pH 7.5).

Subcellular Localization by Immunofluorescent Confocal Microscopy

Mv1Lu cells, plated on gelatin-coated Permanox chamber slides (Nunc), were transfected by the calcium phosphate-DNA precipitation method. Fixation, permealibization, and reaction with the primary and secondary antibodies were described previously (Hoodless et al., 1996). Staining of endogenous SARA and Smad2/3 in Mv1Lu cells was as described previously (Janknecht et al., 1998).

Transcriptional Response Assay

Mv1Lu or HepG2 cells were transiently transfected with CMV-βgal and the indicated constructs using calcium-phosphate transfection as previously described (Labbé et al., 1998). Twenty-four hours after transfection, cells were incubated overnight with or without 50 pM TGFβ. Luciferase activity was measured using the luciferase assay system (Promega) in a Berthold Lumat LB 9501 luminometer and was normalized to β-galactosidase activity.

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GenBank Accession Numbers

The accession numbers for human and *Xenopus* SARA are AF104304 and AF104305, respectively.